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Molecular and Biochemical Analyses of Human Immunodeficiency Virus Type 1 *vpu* Protein

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We have performed a detailed analysis of the biochemical properties of the human immunodeficiency virus (HIV) type 1 *vpu* gene product to elucidate its function during virus replication. Our data suggest that *vpu* is posttranslationally modified by phosphorylation, since a 16-kilodalton phosphoprotein can be specifically immunoprecipitated with both a serum from an HIV-positive individual (HIV-positive serum) and a *vpu*-specific antiserum. In contrast, our results suggest that *vpu* is not glycosylated, even though the protein contains a potential glycosylation site. In vitro translation studies demonstrated that *vpu* is cotranslationally integrated into microsomal membranes, suggesting that *vpu* is an integral membrane protein. While *vpu* was found in significant quantities in virus-producing cells, the protein could not be detected in cell-free culture fluids and is therefore most likely not virion associated. Processing of viral precursor proteins was unaffected by the absence of *vpu*, and no differences were detected in the protein compositions of wild-type and mutant virions. However, virus release from cultures producing *vpu*-defective virus was found to be delayed, resulting in the intracellular accumulation of viral proteins. Our data suggest that *vpu* has a function in the release of virus particles from infected cells.

Like other members of the lentivirus subfamily, human immunodeficiency virus type 1 (HIV-1) contains several open reading frames in addition to the *gag*, *pol*, and *env* open reading frames present in other retroviruses. One of these open reading frames, *vpu*, was only recently shown to encode a 16-kilodalton protein that is expressed in cells infected with HIV-1 but not HIV-2 or simian immunodeficiency virus (3, 13, 19). Elimination of the *vpu* open reading frame results in a decrease in the amount of virus detectable in the culture fluid (19). The predicted amino acid sequence of the *vpu* protein suggests an extremely hydrophobic N terminus, while the C-terminal portion of the 81-amino-acid protein is hydrophilic, containing a high number of charged amino acids. Both the size of *vpu* and its amino acid composition show similarities to those of some small membrane-associated proteins present in orthomyxovirus- and paramyxovirus-infected cells (11, 23). On the basis of these observations, we previously suggested that *vpu* might function as a matrix protein at the level of virus assembly (19). In this study, we provide a detailed characterization of the *vpu* gene product and discuss potential mechanisms for its function in productively infected cells.

MATERIALS AND METHODS

Cells and viruses. The SW480 colon carcinoma cell line (ATCC CCL 228 [1]) was maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. The $CD4^+$ lymphocytic cell line A3.01, which is a hypoxanthine-aminopterin-thymidine-sensitive derivative of the CEM cell line (5), was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Stocks of HIVNL43 virus and the *vpu* mutant virus (HIVNL-U_{35}) were obtained by transfecting DNA of the cloned plasmids pNL43 (1) and pNL-U_{35} (19) into SW480 cells and collecting the supernatants 24 h later. The supernatants were filtered (0.22 μm) to remove contaminating cells. Routinely, 2×10^6 A3.01 cells were infected with up to 2 ml of undiluted virus stock in a total volume of 10 ml. About 50 to 80% of the culture medium was changed every 2 to 3 days, and cells were maintained at a maximum density of 10^6/ml.

Cloned DNAs. The construction of the infectious molecular clone pNL43 has been described previously (1). The *vpu* mutant pNL-U_{35} was constructed by inserting an 8-base-pair *Xho*I linker into the *Ssp*I site (nucleotide position 6153; see Fig. 1) of pNL43 as described recently (19), resulting in a frameshift that leads to a premature termination of *vpu*. The in vitro transcription plasmid pSP-6 has been described elsewhere (19) and contains the *Eco*RI-*Bam*HI fragment from pNL43 (nucleotide positions 5743 to 8465) cloned into the transcription vector pSP65 (14). Prior to transcription, pSP-6 was linearized with *Bam*HI to allow synthesis of runoff transcripts which encode the first translated exon of *tat* (72 amino acids), *vpu* (81 amino acids), and the N-terminal 756 residues of the *env* gene.

Antisera. The HIV-positive human serum used in these studies, obtained from an asymptomatic male donor, strongly reacts with most of the viral proteins, including *vpu* and *tat*, only weakly with *nef*, and not at all with *vif* (unpublished observations). The control human serum was from an HIV-seronegative male donor. The monospecific polyclonal anti-*vpu* antiserum was raised in rabbits by using *vpu* that was synthesized as a fusion protein in an *Escherichia coli* expression system (F. Maldarelli, manuscript in preparation).

In vitro transcription and translation. Runoff transcripts of pSP-6 DNA were synthesized as described previously (19) and used for translation in rabbit reticulocyte lysates. Standard translations were done in a reaction mixture containing 40 μl of reticulocyte lysate (Promega Biotec, Madison, Wis.), 10 μl of [^{35}S]methionine (10 μCi), and 5 μl of RNA. In reaction mixtures containing microsomal membranes, 3 μl of membranes (Promega; 1.2 eq/μl) was added per 55 μl of reaction mixture. Translation reaction mixtures were incubated for 60 min at 30°C. In the experiments in which microsomal membranes were added after translation, reactions were stopped by the addition of RNase A (final

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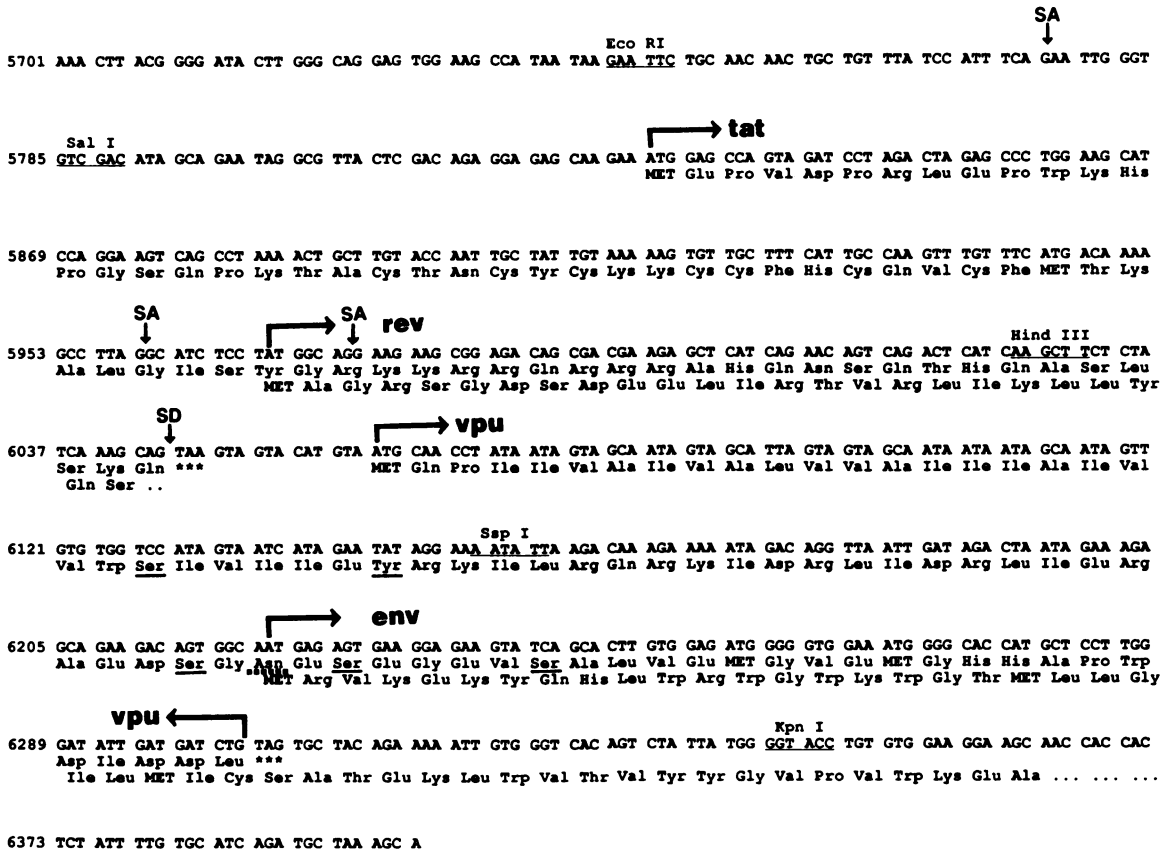


FIG. 1. Structure of the *vpu* gene. The nucleotide sequence in the region of the *vpu* gene of the molecular clone pNL43 (1) is shown. The numbering system used is based on the 5′ end of the proviral DNA as position 1 (17). The translation initiation sites for *tat*, *rev*, *vpu*, and *env* and the 3′ end of the *vpu* gene are indicated by arrows. Potential phosphoamino acids in the *vpu* gene are underlined, and a potential site for N-linked glycosylation is marked (- - -). SA, Splice acceptor site; SD, splice donor site. ***, Stop codon.

concentration, 50 μg/ml). Membranes were added, and incubation was continued for an additional hour at 30°C. Membranes and membrane-associated proteins were pelleted in an air-driven ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) for 60 min at 100,000 × g_{av}. The supernatants were collected, and the pellets were suspended in 500 μl of TN-TN (10 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 0.3% Tween 20, 0.05% Nonidet P-40). The supernatants and the untreated samples were also adjusted to 500 μl with TN-TN prior to immunoprecipitation.

Immunoprecipitation. Protein A-Sepharose was hydrated and washed three times in TN-TN. For each immunoprecipitation reaction, 50 μl (wet volume) of protein A-Sepharose beads was incubated with a 1:20 to 1:500 dilution of antiserum for 1 h at 4°C, washed three times with TN-TN, and incubated with the radiolabeled antigen for an additional hour at 4°C. Unbound material was removed from the beads by washing three times with TN-TN. Immunoprecipitated proteins were solubilized by boiling the samples for 5 min in sample buffer (2% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 1% glycerol, 65 mM Tris hydrochloride [pH 6.8]) and were separated on 12.5% SDS-polyacrylamide gels. Gels were soaked in 1 M sodium salicyclic acid for 20 min and then dried on a gel dryer. The radioactive bands were visualized by fluorography.

Transfection of mammalian cells with cloned DNAs. Cloned DNAs were transfected into SW480 cells by the calcium phosphate precipitation method (8, 9). For each transfection, 15 μg of DNA was adjusted to 250 μM $CaCl_2$ in a total volume of 500 μl with 10 mM Tris hydrochloride [pH 7.6] and was mixed with 500 μl of HBS (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.13], 1.5 mM Na_2HPO_4, 280 mM NaCl). After 30 min, the calcium phosphate-DNA precipitate was added to the SW480 cells. The cells were incubated for 4 h at 37°C and then subjected to a glycerol shock: medium was completely removed and the cells were washed once with phosphate-buffered saline (pH 7.0) before being incubated with 50% HBS–15% glycerol in H_2O for 2.5 min. The cells were washed twice with phosphate-buffered saline and then maintained in Dulbecco modified Eagle medium. In those experiments in which supernatants from the SW480 cells were used as virus stocks for the infection of A3.01 cells, RPMI 1640 medium was added after the glycerol shock.

RESULTS

The *vpu* product is posttranslationally modified. The predicted amino acid sequence of *vpu* contains four serine residues (amino acid positions 23, 52, 56, and 61) and one tyrosine residue (position 29), any of which could be potential sites for phosphorylation (underlined amino acids in Fig. 1), and a single potential site for N-linked glycosylation (-Asn-54-X-Ser-; Fig. 1). To determine whether *vpu* is a phosphoprotein, SW480 cells were transfected with DNA of either the infectious molecular clone pNL43 (1), the *vpu*

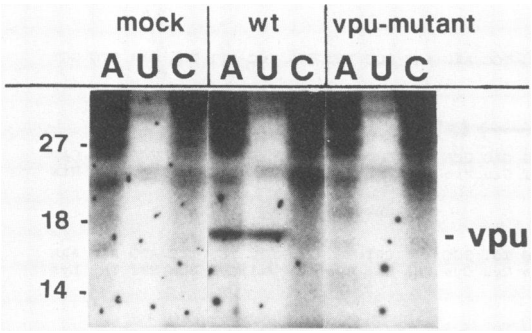


FIG. 2. Phosphorylation of *vpu*. Subconfluent cultures of SW480 cells were transfected with 15 μg of DNA of the infectious molecular clone pNL43 (wt) or the *vpu* mutant pNL-U_{35} (vpu-mutant) or were mock transfected, using the calcium phosphate precipitation protocol (8, 9). Twenty-four hours after transfection, the cells were washed with phosphate-free RPMI 1640 medium and incubated in phosphate-free medium for 30 min at 37°C to deplete the internal phosphate pool. $^{32}P_i$ was added (0.1 mCi/ml), and the cultures were incubated for 3 h at 37°C. The cells were then washed with cold phosphate-buffered saline and lysed in 100 μl of lysis buffer (50 mM Tris hydrochloride [pH 8.0], 5 mM EDTA, 100 mM NaCl, 0.5% CHAPS, 0.2% deoxycholate). HIV-1-specific proteins were immunoprecipitated with an HIV-positive human serum (lanes A), a *vpu*-specific antiserum (lanes U), or a negative control serum (lanes C) and analyzed on a 10 to 20% SDS-polyacrylamide gel prior to autoradiography. A 3-day exposure is shown. Molecular masses (in kilodaltons) are indicated at the left.

mutant pNL-U_{35} (19), or a calcium phosphate precipitate containing no added DNA (mock transfected). Viral proteins were labeled with $^{32}P_i$, immunoprecipitated, and analyzed by gel electrophoresis. Both the HIV-positive serum (Fig. 2, wt, lane A) and antiserum specific for *vpu* (wt, lane U), but not an HIV-negative human control serum (wt, lane C), reacted with a 16-kilodalton protein in lysates from cells transfected with pNL43. This protein was not detected in mock-transfected cells (Fig. 2, mock) or in cells producing the *vpu* mutant virus (Fig. 2, vpu-mutant), indicating that the phosphoprotein detected was indeed *vpu*.

Canine pancreatic microsomal membranes have been used to study glycosylation of proteins in vitro; translation in rabbit reticulocyte lysates containing microsomal membranes can result in N-linked glycosylation (core glycosylation) and be monitored in mobility shift assays on denaturing gels. RNA derived from in vitro transcription of pSP-6 DNA was translated in a rabbit reticulocyte lysate which included microsomal membranes. As described previously (19), this RNA directs the synthesis of *tat* and *vpu* as well as of the initial 756 N-terminal amino acids of the *env* gene (see Fig. 4, diagram). The efficiency of in vitro glycosylation was estimated from the degree of modification of the *env* protein, a well-characterized glycoprotein (21). The HIV-1 *tat* gene does not contain a canonical glycosylation sequence and therefore served as a negative experimental control. Figure 3A shows that the mobilities of both *vpu* and *tat* remained unchanged when in vitro translation was carried out in the presence of microsomal membranes. However, a new, slowly migrating 150-kilodalton band, presumably a glycosylated *env* derivative (compare Fig. 3A, lanes A), was observed. Thus, only the truncated *env* protein, not the HIV *vpu* or *tat* gene product, was glycosylated under these conditions. Although this result does not exclude the possibility that *vpu* is glycosylated in vivo, the similar mobilities of *vpu* synthesized in a rabbit reticulocyte lysate (Fig. 3B, lane 1) or in A3.01 cells during productive viral infection (Fig. 3B, lane 2) is further evidence that *vpu* is not glycosylated.

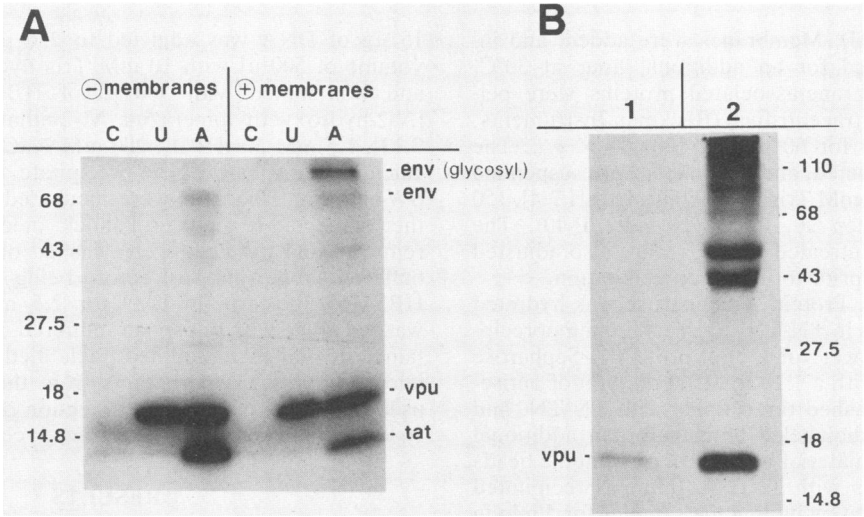


FIG. 3. Attempts to glycosylate *vpu*. (A) Analysis of glycosylation of *vpu* in vitro. Samples of pSP-6 RNA, derived from 0.5 μg of DNA template, were translated in a reaction mixture containing 40 μl of rabbit reticulocyte lysate, 10 μl of [^{35}S]methionine (10 μCi), and 5 μl of RNA. In the reaction mixture containing microsomal membranes (+ membranes), 3 μl (3.5 eq) of canine microsomal membranes was added to the mixture. The translation reaction was performed at 30°C for 1 h. HIV-1-specific proteins were immunoprecipitated with an HIV-positive human serum (lanes A), a *vpu*-specific rabbit antiserum (lanes U), or a negative human serum (lanes C) and separated on a 12.5% SDS-polyacrylamide gel prior to fluorography. The proteins immunoprecipitated with the HIV-positive sera are indicated on the right. (B) Comparison of *vpu* synthesized in vitro and in cell culture. The *vpu* protein was synthesized in a rabbit reticulocyte lysate as described for panel A (no membranes were added) (lane 1). A3.01 cells were infected with HIVNL43 and viral proteins were labeled with [^{35}S]methionine (lane 2), as described in the legend to Fig. 5. Samples of both reaction mixtures were immunoprecipitated with a *vpu*-specific antiserum and analyzed on a 12.5% SDS-polyacrylamide gel. The position of *vpu* in the gel is indicated. Molecular masses (in kilodaltons) are shown.

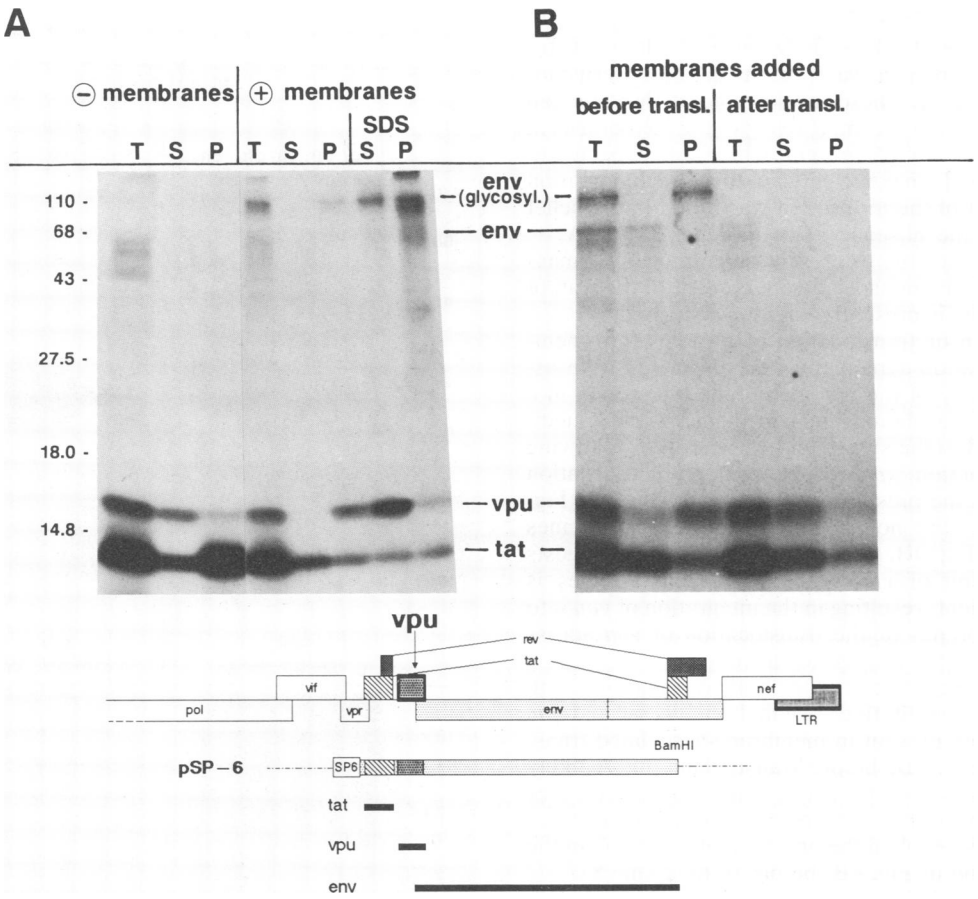


FIG. 4. Analysis of the membrane-associated *vpu*. (A) Transcripts of pSP-6 DNA were translated in the absence (− membranes) or presence (+ membranes) of canine microsomal membranes in rabbit reticulocyte lysates, as described in the legend to Fig. 3. The total volume of each reaction was 60 μl. After 60 min of incubation at 30°C, 30-μl samples were subjected to ultracentrifugation in an airfuge. In a parallel experiment, pSP-6 RNA was translated in the presence of microsomal membranes in a total volume of 50 μl. The reaction conditions were identical to those described above, except that the lysate was adjusted to 0.2% SDS prior to ultracentrifugation. The supernatants were removed, and the pellets were suspended in TN-TN buffer. The individual fractions were immunoprecipitated with an HIV-positive human serum and analyzed on a 12.5% SDS-polyacrylamide gel. Lanes: T, unfractionated lysate; S, supernatant; P, pellet fractions after ultracentrifugation. SDS-treated supernatant and pellet fractions which were electrophoresed are shown in the two lanes at the right (lanes S and P, respectively). (B) Transcripts of pSP-6 DNA were translated in two parallel reaction mixtures, each containing 40 μl of rabbit reticulocyte lysate, 10 μl of [^{35}S]methionine, and 5 μl of RNA. To one of the reaction mixtures, canine microsomal membranes (3.5 eq) were added (before transl.), and both samples were incubated for 60 min at 30°C. The translation reactions were stopped by adding RNase A (50 μg/ml). Microsomal membranes (3.5 eq) were added to the second sample after in vitro translation (after transl.), and both mixtures were incubated for an additional 60 min at 30°C. Samples (30 μl) of each reaction mixture were ultracentrifuged and analyzed as described for panel A. The proteins immunoprecipitated with the HIV-positive human serum are indicated. The general structure of plasmid pSP-6 is depicted at the bottom (19). Proteins encoded on the pSP-6 transcript are indicated (■).

***vpu* is an integral membrane protein.** The analysis of the *vpu* amino acid sequence reveals a stretch of 24 highly hydrophobic amino acids located at the N terminus (amino acids 4 to 27; Fig. 1). Since the membrane anchor domains of integral membrane proteins are characteristically hydrophobic (24), we next investigated whether *vpu* might be such a membrane protein. The in vitro translation of proteins in the presence of microsomal membranes has also proven useful to study integration of proteins into membranes or translocation across membranes (2, 4, 10). Both of these processes can be measured in cosedimentation assays of the translation products (12).

As was described in the previous section, pSP-6 RNA was translated in reticulosyte lysates in the presence or absence of microsomal membranes (Fig. 4A). One-half of each reaction mixture was then centrifuged in an air-driven ultracentrifuge (100,000 × g_{av} for 1 h), and supernatants and pellets were separately recovered. The supernatant (Fig. 4, lanes S), pellet (lanes P), and uncentrifuged (lanes T) samples were immunoprecipitated with an HIV-1-positive human serum and analyzed on an SDS-polyacrylamide gel. In a separate reaction, pSP-6 RNA was translated in the presence of membranes but centrifuged in 0.2% SDS (Fig. 4A, lanes SDS). In the absence of added microsomal membranes, the majority of *vpu* translated in vitro was present in the supernatant fraction (lanes S), indicating that *vpu* does not sediment as a high-molecular-mass structure under these conditions. The small amount of *vpu* present in the pellet fraction following in vitro translation carried out in the absence of microsomal membranes (Fig. 4A, − membranes, lane P) most likely reflects residual contaminating supernatant. In contrast, when pSP-6 RNA was translated in the presence of microsomal membranes, *vpu* as well as the glycosylated, slowly migrating form of envelope sedimented

with the membrane vesicles (Fig. 4A, + membranes, lane P). When SDS was added to the samples prior to ultracentrifugation, virtually all of *vpu* was present in the supernatant (Fig. 4A, SDS, lane S). These results suggest that *vpu* can form high-molecular-mass complexes with the membranes in this in vitro system. The *tat* protein, which was expected to be soluble, behaved unexpectedly in these sedimentation experiments. Most of the *tat* protein was found in the pellet fraction, even in the absence of membranes (Fig. 4A, − membranes). Whether this represents high-molecular-mass complexes of *tat* or its nonspecific attachment to the wall of the centrifuge tubes is presently being investigated.

Since integration or translocation of proteins into membranes is a translation-dependent mechanism (20), we assessed the specificity of the *vpu*-membrane interaction in a series of in vitro translations in which microsomal membranes were added to the system either prior to or following the translation reaction. *vpu* sedimented only if translation was carried out in the presence of membrane vesicles (Fig. 4B, before transl.) but not if the microsomal membranes were added later (Fig. 4B, after transl.). This result suggests that the association of *vpu* with microsomal vesicles is translation dependent, resulting in the integration of *vpu* into microsomal membranes or the translocation of *vpu* across the membranes into the intravesicular lumen.

In this system, the association of both *vpu* and *env* with membranes is not quantitative; residual unglycosylated *env* protein is invariably present in membrane-associated translation reactions (Fig. 4B, before transl., lane T). A likely explanation is that some protein synthesis occurs on free ribosomes even in the presence of microsomal membranes (20). This could also explain the presence of some *vpu* in the supernatant fraction of Fig. 4B (before transl., lane S).

***vpu* is cell-associated.** The potential association of *vpu* with progeny virions was examined by following the intracellular and extracellular distribution of *vpu* in productively infected cells. HIV-infected A3.01 cells were pulse-labeled for 30 min with [^{35}S]methionine near the peak of virus production and chased for 0.5, 1.5, 3, or 6 h in nonradioactive medium. Immunoprecipitates of both cell lysates and cell-free supernatants were separated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography.

vpu was readily detected in cell lysates at all time points after immunoprecipitation with either an HIV-positive serum or a monospecific *vpu* antiserum (Fig. 5). Densitometric scanning revealed that *vpu* was present in levels comparable to or greater than those of gp160^{env} and gp120^{env} (data not shown). Furthermore, the intracellular half-life of *vpu* was estimated to be approximately 3 h. No *vpu* protein was detected in cell-free supernatants, even after a 6-h chase period, when other virion proteins such as p24^{gag}, reverse transcriptase, or integrase were readily detected with the HIV-positive human serum (Fig. 5, top, sup, lane 6h). This result indicates that although *vpu* is relatively abundant inside virus-producing cells, it is not released in detectable quantities into the culture fluid and is very likely not a component of progeny virions.

***vpu* affects the release of viral proteins but not the processing of precursor proteins or the protein composition of virions.** To evaluate the role of *vpu* during HIV replication in T lymphocytes, we studied the kinetics of processing and release of viral proteins in cultures producing wild-type or *vpu* mutant virus by comparing HIV proteins present in cell lysates and cell-free supernatants. A3.01 cells were infected with wild-type and *vpu* mutant virus particles and pulse-labeled with both [^{35}S]methionine and [^{35}S]cysteine. Condi-

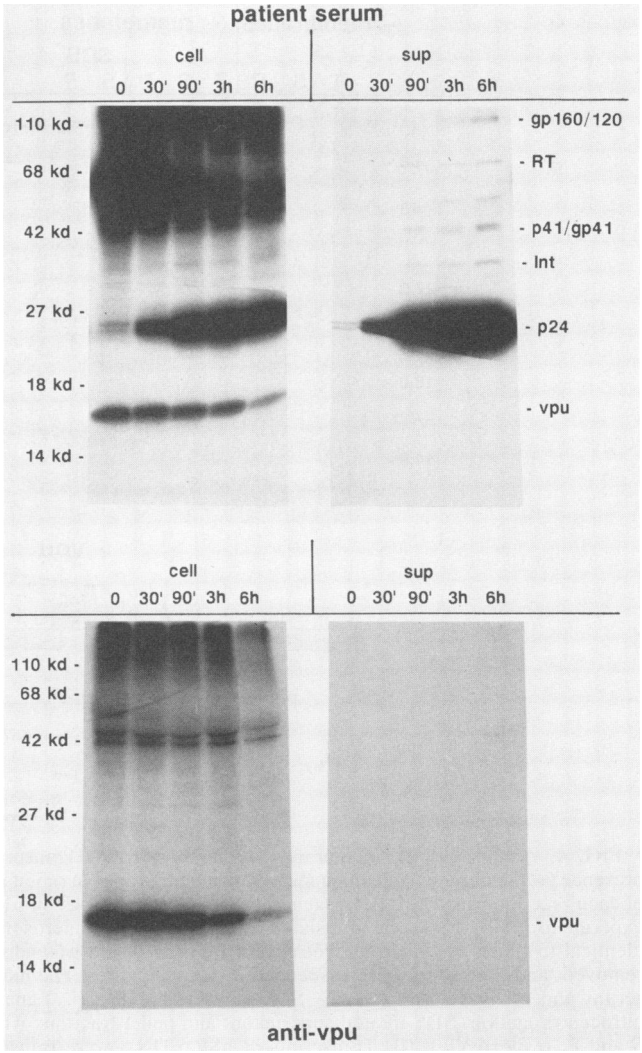
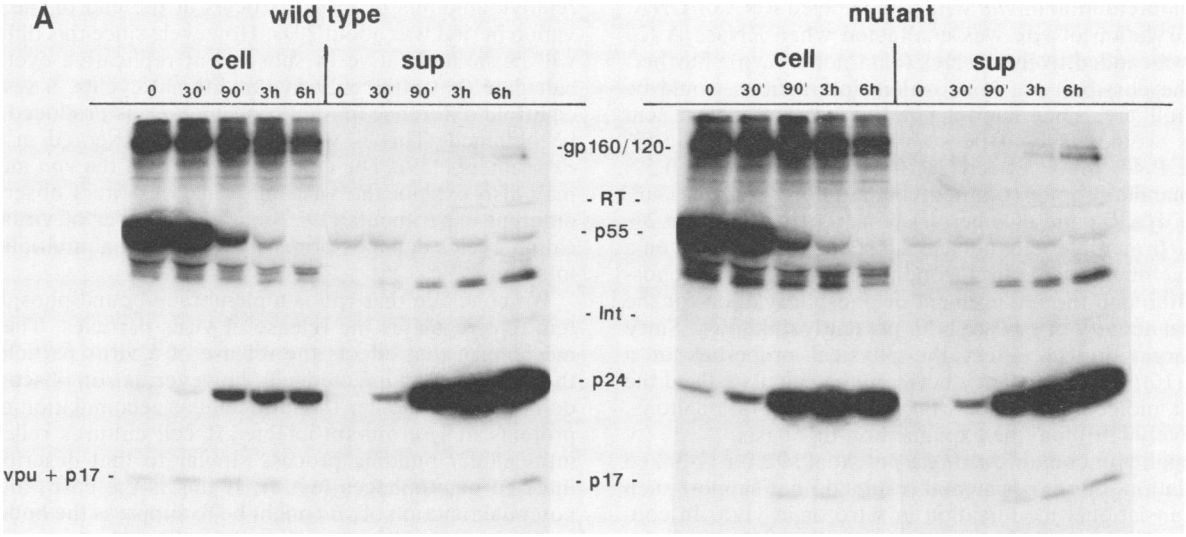


FIG. 5. Detection of the HIV-1 *vpu* protein in cell lysates (cell) but not in culture supernatants (sup). Approximately 2×10^6 A3.01 cells were infected with a virus stock derived from the HIVNL43 cloned proviral DNA (1). Virus production was monitored daily in a reverse transcriptase assay (7, 22). Five days after infection, the cells were washed once in methionine-free RPMI 1640 medium, suspended in 3 ml of the same medium (supplemented with 5% dialyzed fetal calf serum), and incubated for 10 min at 37°C to deplete the internal pool of methionine before [^{35}S]methionine was added (0.4 mCi/ml). Cells were labeled for 30 min at 37°C and, after removal of the isotope, were washed once with complete RPMI 1640 medium (containing 10% fetal calf serum), suspended in 5 ml of complete RPMI 1640 medium, and incubated at 37°C. At the indicated times, 1-ml samples of the cell suspensions were removed; the cells were pelleted and lysed in 120 μl of lysis buffer, as described in the legend to Fig. 2. The supernatants were immediately frozen on dry ice. Samples of the cell lysates (20 μl) and of the supernatants (160 μl) from each time point were immunoprecipitated with either an HIV-positive human serum (patient serum) or a *vpu*-specific antiserum (anti-vpu) and analyzed on 12.5% SDS-polyacrylamide gels. Viral proteins detected in the supernatant fractions with the HIV-positive serum are indicated (top). An overexposure (5 days) of the gel is shown. RT, Reverse transcriptase; Int, integrase.



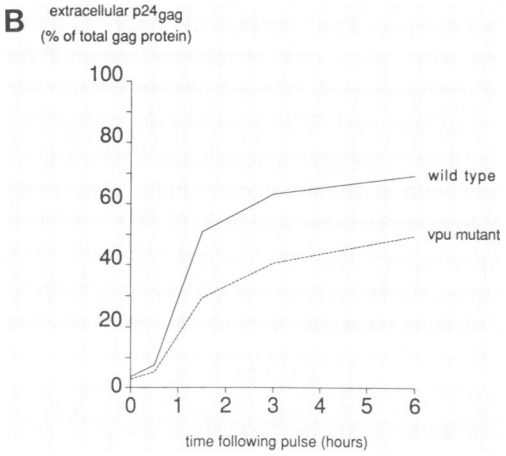


FIG. 6. (A) Kinetics of processing and transport of viral proteins. Approximately 2×10^6 A3.01 cells were infected with either wild-type virus (HIVNL43) or the *vpu* mutant (HIVNL-U_{35}). Near the time of peak virus production (day 5), a pulse-chase experiment was performed as described in the legend to Fig. 5, except that both [^{35}S]cysteine and [^{35}S]methionine were used. Samples of the cell lysates (cell) and the supernatants (sup) were immunoprecipitated with an HIV-positive human serum and analyzed on 12.5% SDS-polyacrylamide gels. An overnight exposure of the gels is shown. Viral proteins are indicated. RT, Reverse transcriptase; Int, integrase. (B) Release of $p24^{gag}$ into culture fluid. All the lanes from the fluorogram shown in panel A were analyzed densitometrically, and the amounts of $p24^{gag}$ in the supernatants of wild-type- and *vpu* mutant-infected cultures were calculated as the percentage of the total $p55^{gag}$ and $p24^{gag}$ protein for any given time point.

tions were chosen to ensure the incorporation of comparable amounts of radioactivity into the HIV proteins synthesized in both cultures. The pulse-labeled cells were chased for 0.5, 1.5, 3, and 6 h, and immunoprecipitates of the cell lysates or cell-free supernatants were analyzed on SDS-polyacrylamide gels.

In both wild-type- and *vpu* mutant-infected cells, the processing of the $p55^{gag}$ precursor occurred with indistinguishable kinetics and was essentially completed within 3 h. The disappearance of $p55^{gag}$ was almost exclusively due to processing into mature *gag* products such as $p24^{gag}$, since only a small amount of unprocessed $p55^{gag}$ was detected in the supernatants of both cultures. Processing of the *env* precursor protein $gp160^{env}$ was not as efficient as that of $p55^{gag}$, and significant amounts of unprocessed $gp160^{env}$ were detected 6 h after synthesis (Fig. 6A, cell, lane 6h). However, no differences in the processing kinetics were observed between the wild type and the *vpu* mutant.

Since processing of the viral precursor proteins did not seem to be affected by the *vpu* mutation, we compared the rates of secretion of virus particles by measuring the release of $p24^{gag}$ into the culture supernatants. Densitometric analysis of $p24^{gag}$ in the fluorogram in Fig. 6A is shown in Fig. 6B. The 100% value was defined as the sum of extracellular plus intracellular $p24^{gag}$ and its precursor $p55^{gag}$ at the indicated time points. It is apparent from Fig. 6B that secretion of $p24^{gag}$ is delayed in cells infected with the *vpu* mutant. More than 50% of $p24^{gag}$ was released within 90 min of synthesis from cells infected with wild-type virus. The secretion of $p24^{gag}$ from cells infected with the *vpu* mutant never reached this level during the 6-h chase period. After 6 h of chase, approximately 50% of $p24^{gag}$ in cultures infected with the *vpu* mutant was still cell associated, whereas more than 70% of the $p24^{gag}$ from the wild-type infection had been released.

It should be noted that $p17^{gag}$, which has been previously shown to be virion associated (15), had a mobility in our gel systems that was indistinguishable from that of *vpu*. However, when proteins were labeled with [^{35}S]methionine (Fig. 5), no incorporation into $p17^{gag}$ occurred because the N-terminal methionine residue is cleaved off during the myristoylation of $p17^{gag}$ (15). In contrast, $p17^{gag}$ was detected in culture supernatants (Fig. 6), since both [^{35}S]methionine and [^{35}S]cysteine were employed.

DISCUSSION

Mutational inactivation of *vpu* has been previously shown to cause a 5- to 10-fold reduction of progeny virus production which is associated with intracellular accumulation of viral proteins (19). Since the mechanism(s) responsible for this phenotype was unclear, a series of biochemical and molecular studies of *vpu* were initiated to elucidate its role during productive viral infection.

The experiments described above show that *vpu* is phosphorylated. Even though we cannot formally rule out a

potential interaction of *vpu* with radiolabeled RNA or DNA, phosphorylation of *vpu* was unaffected when RNase A (25 μg/ml) was added to the lysates (data not shown). Furthermore, the possibility of noncovalent interactions would be highly unlikely, since such a putative protein-nucleic acid complex would have to be stable in a mixture of 0.05% Nonidet P-40, 0.3% Tween 20, 0.2% deoxycholate, 0.5% 3-(3-cholamidopropiopyl-dimethylammonio)-1-propane sulfonate (CHAPS) and also be resistant to being boiled in 2% SDS–5% β-mercaptoethanol and subsequent separation on a protein gel under denaturing conditions. The site(s) of phosphorylation and the requirement of phosphorylation for the biological activity of *vpu* are both presently unknown. Since phosphorylation can affect the physical properties of a protein (16), the discrepancy between the calculated and the apparent molecular masses of *vpu* (9 and 16 kilodaltons, respectively [19]) may be explained on this basis.

Although *vpu* contains a single potential site for N-linked glycosylation, our experimental results do not support such a posttranslational modification in vitro or in vivo. In contrast, we have confirmed a previous speculation (19) by demonstrating the specific, translation-dependent association of *vpu* with microsomal membranes. The microsomal vesicles that were used in this study are derived from the endoplasmic reticulum of pancreatic cells and have proven useful for investigations of the integration or translocation of proteins through membrane structures (20). Our results suggest that *vpu* is synthesized in vivo on membrane-bound ribosomes and either integrates into the membranes of the rough endoplasmic reticulum or is translocated across membranes as a secretory protein. Even though we cannot distinguish between those two mechanisms from the in vitro experiments described above, the preliminary results from protease protection experiments suggest that neither the membrane-associated nor the soluble form of *vpu* is protected from trypsin degradation. In contrast, antibodies specific for *vpu* provide full protection from trypsinization (F. Maldarelli, data not shown), suggesting that at least the 54 C-terminal amino acids of the membrane-bound *vpu*, which include all of the potential trypsin cleavage sites, are exposed on the extraluminal site of membrane vesicles and are susceptible to protease digestion. If *vpu* is translocated across the membranes of the endoplasmic reticulum, it should eventually be secreted. Our failure to detect *vpu* in cell-free supernatants of virus-producing cells is further evidence that *vpu* is not a secretory protein but, rather, an integral membrane protein.

The data accumulated thus far are compatible with HIV *vpu* being functionally important during the assembly or release of progeny virus particles. The results of pulse-chase experiments certainly reveal no differences in the kinetics of $p55^{gag}$ or $gp160^{env}$ precursor protein processing (Fig. 6A). It is unlikely, therefore, that *vpu* is involved in the maturation of viral proteins. Furthermore, since the relative proportions of the different viral proteins in released (supernatant) wild-type and *vpu* mutant particles were indistinguishable (Fig. 6A), we conclude that *vpu* has little, if any, role in determining the protein composition of secreted virions.

In contrast, the pulse-chase experiments suggest that *vpu* exerts its effect during the release of progeny virus particles. Under the conditions employed, the kinetics of wild-type and mutant virus release was monitored over a 6-h period, reflecting what might occur during a single replicative cycle. We found that the secretion of viral proteins, measured as the release of $p24^{gag}$ into the culture fluid, was significantly delayed in the *vpu* mutant infection. The difference in the relative amounts of virus particles at the end of the observation period was about 20%. However, since this difference will be multiplicative in subsequent replicative cycles, we calculate that after only five additional cycles, a seven- to eightfold difference in total progeny virions produced would be obtained. This is within the range observed in earlier experiments (19). The cumulative effect of the *vpu* mutation may also explain the variability in virus titers observed in different experiments (19), since the number of virus replication cycles depends primarily on the initial multiplicity of infection.

We conclude that *vpu* is a membrane-bound phosphoprotein that regulates the release of virus particles. The exact mechanism that effects the release of a virus particle from the surface of an infected cell, however, is still obscure. We do not know whether the intracellular accumulation of viral proteins in *vpu* mutant-infected T-cell cultures reflects an intracellular budding process similar to that described for infected macrophages (6, 18). If this is the case, then one potential function of *vpu* might be to suppress the budding of virions on membranes other than the plasma membrane. Alternatively, the release of virions might involve some interaction of *vpu* with cellular or other viral proteins, explaining why *vpu* is not absolutely required for HIV release, at least in the cell systems analyzed so far. *vpu* could function indirectly by inducing a cellular "releasing factor" or have a direct effect by cooperatively interacting with cellular proteins, thereby accelerating virus release. The association with a cellular factor might also explain the observed intracellular accumulation of HIV virions in macrophages (6, 18), which may lack such a protein. The precise intracellular localization of *vpu* and electron microscopic examination of cells producing mutant virus may provide valuable insights regarding the mechanism of *vpu* function.

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